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International application number: PCT/SE05/000252

International filing date: 23 February 2005 (23.02.2005)

Document type: Certified copy of priority document

Document details: Country/Office: SE  
Number: 0401942-8  
Filing date: 28 July 2004 (28.07.2004)

Date of receipt at the International Bureau: 08 March 2005 (08.03.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland  
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(21) Patentansökningsnummer 0401942-8  
Patent application number

(86) Ingivningsdatum 2004-07-28  
Date of filing

Stockholm, 2005-02-28

För Patent- och registreringsverket  
For the Patent- and Registration Office

  
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Avgift  
Fee

PPU04-07-28

## NEW ANTIMICROBIAL PEPTIDE COMPLEXES

### ABSTRACT

The invention relates to new stable colloidal complexes between a charged antimicrobial peptide belonging to the cathelicidin family and a bilayer-forming polar lipid. The new complex can be used as a drug delivery system for the charged antimicrobial peptide in the treatment of infections, in wound healing and in other diseases with a deficiency in antimicrobial activity.

### FIELD OF THE INVENTION

The present invention relates to new stable antimicrobial peptide complexes comprising a cathelicidin peptide and a neutral bilayer-forming polar lipid in an aqueous solution. More specifically, the present invention refers to the use of new complexes as drug delivery systems for antimicrobial peptides in infections, in wound healing and in other diseases with a deficiency in antimicrobial activity. The novel drug delivery system retards degradation of the peptide, reduces toxicity and prevents adsorption of the drug to non-biological surfaces. The system can most preferably be used for delivery of the antimicrobial peptide at biological surfaces.

### BACKGROUND OF THE INVENTION

A major goal in the pharmacological arts has been the development of methods and compositions to facilitate the specific delivery of therapeutics to the appropriate cells and tissues that would benefit from such treatment, and the avoidance of the general physiological effects of the inappropriate delivery of such agents to other cells or tissues of the body. This is particularly important in the delivery of antimicrobial and antiviral compounds. These compounds typically have immunogenic or cytotoxic effects that damage or destroy uninfected cells as well as infected cells. In addition, certain compounds, drugs or agents are "activated" or chemically modified by an enzymatic or chemical activity specific for infected cells, in which an activated form of the compounds are particularly toxic. Thus, an efficient delivery system which would enable the delivery of such compounds, particularly said "activated" forms thereof, specifically to infected cells would increase the efficacy of treatment, overcome drug resistance, reduce the associated "side effects" of such drug treatments.



Numerous methods for enhancing the activity and the specificity of drug action have been proposed. One method involves linking the therapeutic agent to a ligand which has an affinity for a receptor, expressed on the desired target cell surface. Using this approach antimicrobial and antiviral compounds are intended to adhere to the target cell following formation of a ligand-receptor complex on the cell surface. Entry into the cell could then follow as the result of internalization of ligand-receptor complexes. Following internalization, the antimicrobial or antiviral compounds may then exert therapeutic effects directly on the cell.

The approach has many drawbacks and limitations. One is the concentration of the drug at the site will be limited although the use of for example lipid conjugates have improved the concentration. See for example US. Pat. No. 5,484,809, which discloses taxol and taxol derivatives conjugated to phospholipids. Another drawback is the modification of the compound with a ligand, which may change the metabolism of the drug-ligand complex. The present invention is based on the manufacture of stable peptide-polar lipid complexes, where the peptide is linked to the lipid through non-covalent forces and where the polar lipid is a bilayer-forming lipid. This complex has many advantages in comparison to other delivery systems known in the art.

With bilayer is normally meant the lamellar arrangements of polar lipids in water. The acyl chains form the internal hydrophobic part and the polar head-groups the hydrophilic part of the bilayer. As examples of such polar bilayer-forming lipids, either of natural or synthetic origin, can be mentioned phosphatidylcholine, phosphatidylglycerol, digalactosyl-diacylglycerol, sphingomyelin and the like. Depending on the concentration of said polar lipids in polar solvents such as water stable peptide complexes can be formed, which according to the present invention can be kept stored for days.

Antimicrobial peptides are highly charged effector molecules of the innate immune system, which serve to protect the host against potentially harmful microorganisms. They are conserved through evolution and are widespread in nature. In human, only a handful has been identified so far among which the defensins and the human cathelicidin antimicrobial peptide hCAP18 have been implicated in epithelial defence. It has been proposed that cationic peptides interact with microorganisms by binding to their negatively charged surfaces.

It has also been shown that up-regulation of hCAP18 and/or by adding the LL-37 peptide will stimulate proliferation of normal epithelial and stromal cells, suggesting that normal wound healing and epithelial regeneration could be enhanced using this antimicrobial peptide.

WO 96/08508 relates to the human polypeptide FALL-39, as well as to pharmaceutical compositions containing said peptide and having an antimicrobial activity against bacteria. The peptide was named FALL-39 after the first four amino acid residues and consisted of the 39 amino acid C-terminal part of a proprotein concomitantly identified by three separate groups (Cowland *et al.*, *FEBS*, 1995; Agerberth *et al.*, *Proc Natl Acad Sci USA* 1995; Larrick *et al.*, *FEBS Letters* 1996). The peptide was shown to have potent antimicrobial activity against both gram-positive and gram-negative bacteria. Further characterization of the C-terminal peptide demonstrated a shorter sequence comprising 37 amino acids excluding the first two (FA) resulting in LL-37, which is the accepted current designation (Gudmundsson *et al.*, *Eur J Biochem* 238:325-332, 1996). The proprotein was named hCAP18, Human Cationic Antimicrobial protein, and is a member of the cathelicidin family of proteins consisting of cathelin, which has been conserved through evolution and a C-terminal part, variable in different species. In man, hCAP18 is the only member of this protein family, whereas in other species, such as mouse and pig, there are several members. The C-terminal peptide LL-37 is thought to function extracellularly and there is no evidence for intracellular cleavage of the pro-protein. hCAP18/LL-37 is present in leukocytes and in barrier organs such as skin, mucous membranes, respiratory epithelium and reproductive organs. The localization of hCAP18/LL-37 to barrier epithelia seems to be consistent with a protective role for the peptide in preventing local infection and systemic microbial invasion. LL-37 is described as a cysteine-free peptide that can adopt an amphiphatic, or in other words amphiphilic,  $\alpha$ -helical conformation. A high cationicity in combination with a stabilized amphiphatic  $\alpha$ -helical structure seems to be required for the antimicrobial effect of such peptides against gram-positive bacteria and fungi, as has been shown experimentally (Gianga-Spero *et al.*, *Eur J Biochem* 268:5589-5600, 2001).

Although a therapeutic use of antimicrobial peptides, in particular LL-37, has been suggested, this has so far not been realized. At high concentrations of the peptide, LL-37 exerts a cytotoxic effect. The potential cytotoxic effects exerted by LL-37 are, however, inhibited in the presence of serum, but pharmaceutical formulations containing serum should be avoided

due to risk for transmitting diseases, restricted accessibility and high costs. It has recently been shown that the cytotoxic effect can also be inhibited by CPL-Galactolipid, which is a polar and neutral bilayer-forming lipid material.

It has recently been demonstrated (PCT/SE04/00111) that mixtures consisting of the peptide LL-37 and galactolipids unexpectedly formed stable, clear colloidal solutions at certain weight ratios. The appearance of the resulting solutions indicated that the peptide and the lipid formed complexes that were considerably smaller in size than corresponding peptide-free liposomes. Colloidal solutions are per definition thermodynamically stable, and unlike liposomal dispersions, they do not separate on storing. Furthermore, it was shown in PCT/SE04/00111 that the *in vitro* cytotoxicity of LL-37 was reduced when complexed with galactolipids.

The present invention demonstrates that other peptides belonging to the cathelicidin family of peptides also form stable solutions, provided that the peptide has a molecular weight between 2.5-5 kDa (free base).

#### DESCRIPTION OF THE INVENTION

The use of galactolipid-based liposomes in pharmaceutical applications has been described in WO 95/20944. This application does not disclose the use of galactolipids in combination with peptides and proteins in general, particularly not for forming complexes in solution, i.e. clear colloidal solutions which are physically stable.

The present invention discloses stable galactolipid-peptide colloidal solutions, where the galactolipid and the peptide form a complex at certain weight ratios. The peptide shall have a molecular weight between 2.5-5 kDa to form a stable complex which can be stored for days. The peptide shall also be cationic. The peptide-lipid weight ratio shall be between 1:5 to 1:50. Suitable counterions are acetate, chloride, etc, for a positively charged peptide. Preferred peptides are LL-25, LL-26, LL-27, LL-28, LL-29, LL-30, LL-31, LL-32, LL-33, LL-34, LL-35, LL-36, LL-37 and LL-38.

The galactolipid from Sigma was obtained by purification from whole wheat flour. Galactolipids from any source including synthetic compounds can be used in the invention. The preferred tested lipid was a galactolipid material referred to as CPL-Galactolipid,

manufactured by LTP Lipid Technologies Provider AB, Sweden. This is a purified galactolipid fraction from oats.

A suitable aqueous medium for the complexes is phosphate-buffered saline (PBS; 50 mM sodium phosphate, 150 mM NaCl, pH 7.4). However, any other aqueous solution with comparable ionic strength and appropriate pH may be used for the preparation.

The composition can in addition comprise pharmaceutically acceptable excipients, such as a preservative to prevent microbial growth in the composition, antioxidants, additional isotonicity agents, colouring agents, stabilising agents such as non-ionic surfactants and hydrophilic polymers, and the like.

The invention is not limited to the use of a galactolipid, such as CPL-Galactolipid from oats. Other polar bilayer-forming lipids can be used, such as phospholipids (e.g. phosphatidylcholine, phosphatidylglycerol), or sphingolipids (e.g. sphingomyelin).

#### DETAILED DESCRIPTION OF THE INVENTION

The LL-20, LL-25, LL-37 and LL-38 peptides were synthesized using solid phase synthesis with the 9-fluorenylmethoxycarbonyl / tert-butyl strategy. The crude peptides, as the trifluoroacetate salts, were purified by HPLC and finally isolated by lyophilization. The purity was determined by means of HPLC. Analysis of composition of amino acids showed that the relative amounts of each amino acid corresponded with the theoretical values for the respective peptide. The antimicrobial activity of the peptides were tested using an inhibition assay; see below.

The CPL-Galactolipid, which is based on an extraction of natural galactolipids from oats, is today used in dermatological creams and has been shown to be well tolerated, and to have good absorption properties. CPL-Galactolipid is stable at ambient temperature. Based on these data it can be concluded that the complex can be administered topically during long periods of time, for example in wound healing.

#### General procedure for forming a stable peptide-galactolipid complex in aqueous solution

The peptide and the galactolipid are weighed in a 100 ml glass flask and then PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.4) is added. The sample is vigorously shaken, using a suitable shaker at high speed, for 1-2 h or until the mixture has become clear, and is then allowed to equilibrate and settle for about 30 min at room temperature. It should be noted that the procedure does not involve the use of ultrasonicators, high-speed mixers (ultra-turrax), high-pressure homogenisers, or other processing equipment, which is a clear advantage from a technical and economical point of view. Furthermore, it does not require heat treatment, which makes it possible to prepare compositions containing heat sensitive bioactive compounds.

The colloidal nature of the composition makes it possible to prepare it aseptically by employing a final sterile filtration step. This is especially advantageous if the composition contains a bioactive molecule which is heat sensitive and thus not possible to heat sterilise.

Example 1. Preparation of aqueous mixtures comprising a mixture of a cathelicidin-derived peptide and a lipid material

Samples of LL-20, LL-25, LL-37 and LL-38 as trifluoroacetate salts and a polar, bilayer-forming lipid material, CPL-Galactolipid, were prepared using the following amounts.

Table 1

Sample No	LL-20 (MW 2440 kDa)	LL-25 (MW 3065 kDa)	LL-37 (MW 4493 kDa)	LL-38 (MW 4598 kDa)	Appearance
1 (CPL-Galactolipid in PBS)	-	-	-	-	Turbid dispersion
2	52 ppm	-	-	-	Cloudy dispersion
3	98 ppm	-	-	-	Cloudy dispersion
4	-	68 ppm	-	-	Almost clear solution
5	-	98 ppm	-	-	Almost clear solution
6	-	-	44 ppm	-	Slightly turbid dispersion
7	-	-	98 ppm	-	Almost clear solution



8	-	-	213 ppm	-	Clear solution
9	-	-	-	50 ppm	Slightly turbid dispersion
10	-	-	-	102 ppm	Almost clear solution

The peptide mixtures, all containing 0.20 % CPL-Galactolipid and PBS (phosphate-buffered saline, pH 7.4), were prepared according to the general procedure described above. Ocular inspections were made after 2 h and 2 days of storage at room temperature of the mixtures. These inspections revealed that LL-25, LL-37 and LL-38 in the concentration ranges of 68-98 ppm, 98-213 ppm and 50-102 ppm, respectively, resulted in clear or almost clear solutions. The LL-20 mixture showed large sediments in the investigated concentration range.

The interactions between the charged antimicrobial peptide and the neutral lipid are supposed to be sufficiently strong to accomplish a stabilization of the peptide but weak enough to release the peptide from the complex once it has been delivered to the site of action as shown in wound healing experiments.

The data thus demonstrate that stable complexes are formed between the cationic peptide and CPL-Galactolipid only if the peptide has a molecular weight > 2.5 kDa.

#### Example 2. Preparation of aqueous mixtures comprising varying contents of LL-37 peptide and varying contents of galactolipid

A stock solution of LL-37 peptide (acetate salt) in PBS, 995 ppm, and a stock solution of CPL-Galactolipid, 1.00 %, in PBS were prepared. Aliquots of the stock solutions plus additional PBS were mixed in 20 ml glass vials with rubber stoppers and aluminum caps. The compositions of the mixtures are presented in Table 2. After equilibration at room temperature for 1 h, the vials were shaken in horizontal position on an ST mixer (type B1, E. Büchler, Tübingen), set at 7.5 (corresponding to an approximate frequency of 190/min), for 1 h. The mixtures were then allowed to equilibrate and settle over night at room temperature. The appearances of the mixtures after one and five days at 4°C were evaluated as: clear colloidal, slightly turbid, turbid, milky, and the results are summarized in Table 2.

Table 2.

Sample number	LL-37 (ppm)	Galacto-lipid (%)	Peptide: Lipid (w/w)	Appearance after 1 day	Appearance after 5 days
01	247	0.135	1:5.5	turbid dispersion, sediment	turbid dispersion, sediment
02	181	0.133	1:7.4	clear colloidal solution	clear colloidal solution, slight sediment
03	116	0.133	1:11	clear colloidal solution	clear colloidal solution
04	50.5	0.135	1:27	clear colloidal solution	clear colloidal solution
05	16.5	0.133	1:81	slightly turbid dispersion, homogeneous	slightly turbid dispersion, homogeneous
06	8.2	0.135	1:165	turbid dispersion, homogeneous	turbid dispersion, homogeneous
07	-	0.133	-	turbid dispersion, homogeneous	turbid dispersion, homogeneous
08	248	0.266	1:11	clear colloidal solution	clear colloidal solution, slight sediment
09	182	0.267	1:15	clear colloidal solution	clear colloidal solution
10	116	0.266	1:23	clear colloidal solution	clear colloidal solution
11	49.8	0.268	1:54	slightly turbid dispersion, homogeneous	slightly turbid dispersion, homogeneous
12	17.1	0.266	1:156	slightly turbid dispersion, homogeneous	slightly turbid dispersion, homogeneous
13	8.9	0.265	1:298	slightly turbid dispersion, homogeneous	slightly turbid dispersion, homogeneous
14	-	0.265	-	slightly turbid dispersion, homogeneous	slightly turbid dispersion, homogeneous
15	247	0.532	1:22	clear colloidal solution	clear colloidal solution
16	182	0.532	1:29	slightly turbid dispersion, homogeneous	slightly turbid dispersion, homogeneous
17	116	0.533	1:46	turbid dispersion, homogeneous	turbid dispersion, homogeneous
18	49.2	0.533	1:108	turbid dispersion, homogeneous	turbid dispersion, homogeneous
19	16.5	0.534	1:324	turbid dispersion, homogeneous	turbid dispersion, homogeneous
20	8.2	0.532	1:649	turbid dispersion, homogeneous	turbid dispersion, homogeneous
21	-	0.533	-	turbid dispersion, homogeneous	turbid dispersion, homogeneous

22	248	0.799	1:32	turbid dispersion, homogeneous	turbid dispersion, slight sediment
23	182	0.802	1:44	milky dispersion, homogeneous	milky dispersion, slight sediment
24	115	0.801	1:70	milky dispersion, homogeneous	milky dispersion, slight sediment
25	50.1	0.799	1:159	milky dispersion, homogeneous	milky dispersion, slight sediment
26	16.8	0.799	1:476	milky dispersion, homogeneous	milky dispersion, slight sediment
27	8.6	0.798	1:928	milky dispersion, homogeneous	milky dispersion, slight sediment
28	-	0.798	-	milky dispersion, homogeneous	milky dispersion, slight sediment

It is clear that certain ratios of LL-37 peptide and galactolipid give rise to an appearance in solution, which indicates the presence of small complexes, smaller in size than particles of the corresponding samples without LL-37. A clear solution indicates a superior colloidal stability.

Example 3. Test of antimicrobial activity of LL-20 and LL-25

The antimicrobial activity of LL-37 has been shown by others in previous studies. The antimicrobial activity was tested using an inhibition zone assay. As a test bacterium, *Bacillus Megaterium* was used. The following data was obtained.

Table 3

Sample No.		Mean (mm)
1	0.203 % CPL-Galactolipid (GL) in PBS	neg
2	68 ppm LL-25 + 0.200 % GL in PBS	7.5
3	98 ppm LL-25 + 0.200 % GL in PBS	7.9
4	52 ppm LL-20 + 0.203 % GL in PBS	neg
5	98 ppm LL-20 + 0.200 % GL in PBS	neg
6	100 ppm LL-25 in PBS	8.8

The data shows that LL-25 showed an antimicrobial activity at a concentration of 68 ppm. It was also shown that LL-25 exhibit activity using the complex with CPL-Galactolipid. The complex with LL-20 had no antimicrobial activity.

The present invention is not limited in scope to the described examples. Various modifications of the invention will be apparent to those skilled in the art. It is thus anticipated that it should be possible to form similar complexes based on other polar bilayer-forming lipids using other antimicrobial peptides having molecular weights less than 5 kDa, and being cationic. The optimal conditions, that is, weight ratio of compound to lipid and total concentration of the two ingredients are obtained by experiments as shown above. The aqueous solution should have an appropriate composition, ionic strength and pH. The best composition for each unique antimicrobial peptide and galactolipid mixture is thus established and validated by means of the technically simple procedure described above.

#### CLAIMS

1. A peptide-lipid complex in an aqueous solution having a weight ratio of 1:5 – 1:50, preferably 1:10 – 1:27, wherein said peptide is a cationic antimicrobial peptide, having a molecular weight of 2.5-5 kDa and where the lipid is a bilayer-forming lipid.
2. The complex according to claim 1 where the peptide is derived from the cathelicidin family of peptides.
3. The complex according to claim 1 where the peptide is in the form of a pharmaceutically acceptable salt.
4. The complex according to claim 1 where the peptide is LL-25, LL-26, LL-27, LL-28, LL-29, LL-30, LL-31, LL-32, LL-33, LL-34, LL-35, LL-36, LL-37 or LL-38.
5. The complex according to preceding claims where the polar bilayer-forming lipid is a galactolipid.
6. The complex according to claim 1 where the galactolipid is CPL-Galactolipid.
7. The complex according to any of claims 1-6, comprising the peptide LL-25 and a galactolipid material.
8. The complex according to any of claims 1-6, comprising the peptide LL-37 and a galactolipid material.
9. The complex according to any of claims 1-8 where the peptide is in the form of an acetate salt.
10. A method of preparing a polar bilayer-forming peptide-lipid complex which comprises:

- providing a cationic antimicrobial peptide and a polar bilayer-forming lipid;
- adding a buffer solution;
- mixing the components with a mixer until the solution is clear and optionally sterilizing the solution by sterile filtration.

11. A method according to claim 8 where the polar bilayer-forming lipid is a galactolipid.
12. Use of an antimicrobial peptide derived from the cathelicidin family of peptides and a polar bilayer-forming lipid for the manufacture of a peptide-lipid complex as a medicament for treatment of infections, wound healing or other diseases with a deficiency in antimicrobial activity.
13. Use of a peptide-lipid complex according to any of claims 1-8 for the manufacture of a medicament for topical treatment of infections, wounds, atopic eczema and other conditions deficient in antimicrobial activity and/or angiogenesis.

PRV04-07-28